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Published in:
Archives of Microbiology

DOI:
10.1007/BF00417176

Arch. Microbiol. (1981) 129:35–41

Archives of
Microbiology

Biogenesis and Turnover of Peroxisomes Involved in the Concurrent Oxidation of Methanol and Methylamine in *Hansenula polymorpha*

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Abstract. Growth of *Hansenula polymorpha* in shake flasks and chemostat cultures in the presence of methanol as the sole source of carbon and methylamine as the sole source of nitrogen was associated with the development of peroxisomes in the cells. The organelles were involved in the concurrent oxidation of these two compounds, since they contained both alcohol oxidase and amine oxidase, which are key enzymes in methanol and methylamine metabolism, respectively. In addition catalase was present. Peroxisomes with a completely crystalline substructure were observed in methanol-limited chemostat-grown cells. Amine oxidase probably formed an integral part of these crystalloids, whereas catalase was present in a freely diffusable form.

Transfer of cells, grown in a methanol-limited chemostat in the presence of methylamine into glucose/ammonium sulphate media resulted in the loss of both alcohol oxidase and amine oxidase activity from the cells. This process was associated with degradation of the crystalline peroxisomes. However, when cells were transferred into glucose/methylamine media, amine oxidase activity only declined during 2 h after the transfer and thereafter increased again. This subsequent rise in amine oxidase activity was associated with the development of new peroxisomes in the cells in which degradation of the crystalline peroxisomes, originally present, continued. These newly formed organelles probably originated from peroxisomes which had not been affected by degradation. When in the methanol-limited chemostat methylamine was replaced by ammonium sulphate, repression of the synthesis of amine oxidase was observed. However, inactivation of this enzyme or degradation of peroxisomes was not detected. The decrease of amine oxidase activity in the culture was accounted for by dilution of enzyme as a result of growth and washout.

Key words: Peroxisome – Methanol – Methylamine – Yeast – *Hansenula polymorpha* – Alcohol oxidase – Amine oxidase – Catalase – Catabolite inactivation – Turnover – Cytochemical localization

In fungi peroxisomes are known to play a key role in the oxidative metabolism of a number of different carbon sources (Fukui and Tanaka 1979; van Dijken and Veenhuis 1980). The importance of these organelles in the metabolism of methanol by yeasts may be illustrated by the following example. Transfer of glucose-grown cells of the yeast *Hansenula polymorpha* into methanol-containing media leads to the development of a number of peroxisomes in these cells before growth under the new conditions is resumed at a measurable rate. It has been shown that these organelles originate from the small peroxisomes, originally present in the glucose-grown cells by a process of growth and division (Veenhuis et al. 1979a). They possess, depending on growth conditions, a partly or completely crystalline substructure, and contain as the main constituents alcohol oxidase and catalase (Veenhuis et al. 1978a). Both enzymes play a key role in methanol metabolism in this yeast (van Dijken et al. 1975; Roggenkamp et al. 1975).

The process of peroxisome development can be readily reversed. For instance, transfer of methanol-grown cells of *H. polymorpha* into glucose-containing media, in which the synthesis of alcohol oxidase is repressed (Eggeling and Sahm 1978; Egli et al. 1980), led to a rapid inactivation of the enzymes involved in methanol oxidation and degradation of the peroxisomes present in these cells (Bormann and Sahm 1978; Veenhuis et al. 1978b).

Recently, Zwart et al. (1980) demonstrated that yeast peroxisomes may also be involved in the metabolism of a number of nitrogen compounds when these were used as a sole source of nitrogen for growth. These workers reported that growth of *H. polymorpha* and *C. utilis* in media supplemented with glucose in the presence of methylamine as the sole nitrogen source was accompanied by the development in the cells of peroxisomes which contained amine oxidase, a key enzyme in the metabolism of methylamine in these organisms.

These results prompted us to study the role of peroxisomes in yeasts grown in media supplemented with methanol and methylamine, compounds which are both metabolized by peroxisome-borne enzymes. This paper describes the development of large peroxisomes in cells of the yeast *H. polymorpha*, grown under such conditions. They contained both alcohol oxidase and amine oxidase in the same organelle. The ultrastructural and physiological events observed upon transfer of such cells into media in which the activity of either alcohol oxidase, amine oxidase or both is no longer required for growth, are also described.

Materials and Methods

Microorganism and Cultivation. Hansenula polymorpha de Morais et Maya 4732 was used in all experiments. The organism was grown in methanol-limited chemostat cultures at 37° C in the mineral medium of van Dijken et al. (1976) or in batch cultures (Veenhuis et al. 1980), containing 0.5 % (v/v)

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Table 1. Enzyme activities in shake flask cultures of *H. polymorpha*, transferred from glucose/ammonium sulphate into media containing methanol as the C-source and ammonium sulphate or methylamine as the N-source or during growth in a methanol-limited chemostat ($D = 0.088\,h^{-1}$) with methylamine as the N-source and after transfer of the culture to ammonium sulphate instead of methylamine as the N-source. Cells from shake flask cultures were harvested in the exponential phase of growth. Chemostat-grown cells, after transfer to ammonium sulphate as the N-source, were harvested after 3 volumes changes. Oxidase activities are expressed as $\mu mol\ O_2 \cdot min^{-1} \cdot mg\ protein^{-1}$, catalase activity as $\Delta E_{240} \cdot min^{-1} \cdot mg\ protein^{-1}$. The number of peroxisomes is given as the average number per section, volume densities are expressed as percentage of the cytoplasmic volume

Growth conditions	Specific activities of			Number of peroxisomes	Volume density of	
	alcohol oxidase	amine oxidase	catalase		peroxisomes	vacuole
Glucose/$(NH_4)_2SO_4$ (OD_{663} = 1.0)	0	0	10.8	0.04	0.1	
Methanol/$(NH_4)_2SO_4$	1.18	0	39.8	1.2	19.8	
Methanol/methylamine	1.17	$7.8 \cdot 10^{-3}$	34.0	1.3	18.0	
Chemostat ($D = 0.088\,h^{-1}$) methanol/methylamine	2.94	$17.8 \cdot 10^{-3}$	69.9	3.2	40.6	11.9
Chemostat ($D = 0.088\,h^{-1}$) methanol/$(NH_4)_2SO_4$	3.70	$1.6 \cdot 10^{-3}$	78.0	3.3	50.6	3.6

methanol and 0.25% (w/v) methylamine or 0.25% (w/v) $(NH_4)_2SO_4$ as the nitrogen source. Batch cultures were inoculated with cells, growing exponentially in media containing glucose and $(NH_4)_2SO_4$, as described previously (Veenhuis et al. 1979a).

Transfer Experiments. The experiments were performed with cells from methanol-limited chemostat cultures ($D = 0.088\,h^{-1}$), growing in a medium containing 0.5% (v/v) methanol and 0.25% (w/v) methylamine, as described above. The following experiments were performed: (a) Change of the nitrogen source in the medium input. In these experiments methylamine in the reservoir was replaced by 0.25% (w/v) $(NH_4)_2SO_4$ as the nitrogen source. (b) Change of the carbon source. In these experiments chemostat-grown cells were placed in Erlenmeyer flasks, containing the mineral medium described above, supplemented with 0.5% (w/v) glucose and 0.25% (w/v) methylamine. The cultures were inoculated to an optical density (OD_{663}) of 0.2 and incubated at 37° C. (c) Change of both the carbon and the nitrogen source. These experiments were performed as described in (b), except that, instead of methylamine the media contained 0.25% (w/v) $(NH_4)_2SO_4$ as the nitrogen source.

Enzyme Assays. The preparation of cell-free extracts and the estimation of alcohol oxidase activity was as described by van Dijken et al. (1976). Amine oxidase activity was determined as described previously (Zwart et al. 1980). Oxidase activities are expressed as μmol of oxygen consumed · min^{-1} · mg $protein^{-1}$. Catalase was assayed by the spectrophotometric method of Lück (1963). Catalase activity is expressed as $\Delta E_{240} \cdot min^{-1} \cdot mg\ protein^{-1}$. Protein concentrations in cell-free extracts were determined by the method of Lowry et al. (1951), using bovine serum albumin as the standard.

Preparation of Spheroplasts. Spheroplasts were prepared by treatment of whole cells with "Zymolyase" (Kitamura et al. 1971) for 5–20 min at 37° C according to the procedure of Osumi et al. (1975). For osmotic shock treatment, a suspension of spheroplasts was centrifuged and the pellet resuspended in 50 mM K-phosphate buffer pH 7.2 for 1 min before fixation with glutaraldehyde.

Cytochemical Staining Techniques. Cytochemical staining procedures for the detection and localization of catalase and oxidase activities were performed as described previously (Veenhuis et al. 1976). Incubations for the localization of amine oxidase activity were performed at pH 8.0, using 10 mM methylamine as the substrate (Zwart et al. 1980).

Preparation of Ultrathin Cryo-Sections. For ultracryotomy cells grown in the chemostat were fixed in 6% glutaraldehyde in 0.1 M Na-cacodylate buffer pH 7.2 for 15 min at 0° C. Sections were cut with a glass knife using a LKB-cryokit according to the techniques described by Tokoyashu (1978). The sections were collected on Formvar-carbon coated grids, washed with 0.1 M ammonium acetate and water and subsequently stained with 0.5% uranyl acetate. Octadecanol, dissolved in 100% hexane, was used as a wetting agent (Gordon 1972).

Fixation and Postfixation Techniques. Whole cells were fixed in 1.5% $KMnO_4$ for 20 min at room temperature. Spheroplasts were fixed in 6% glutaraldehyde in 0.1 M Na-cacodylate buffer pH 7.2 for 30 min at 0° C. Postfixation of spheroplasts – and also after cytochemical staining techniques – was performed in a solution of 1% OsO_4 and 2.5% $K_2Cr_2O_7$ in 0.1 M Na-cacodylate buffer, pH 7.2 for 45 min at room temperature. After dehydration in a graded alcohol series the material was embedded in Epon 812. Ultrathin sections were cut with a diamond knife and examined in a Philips EM 300.

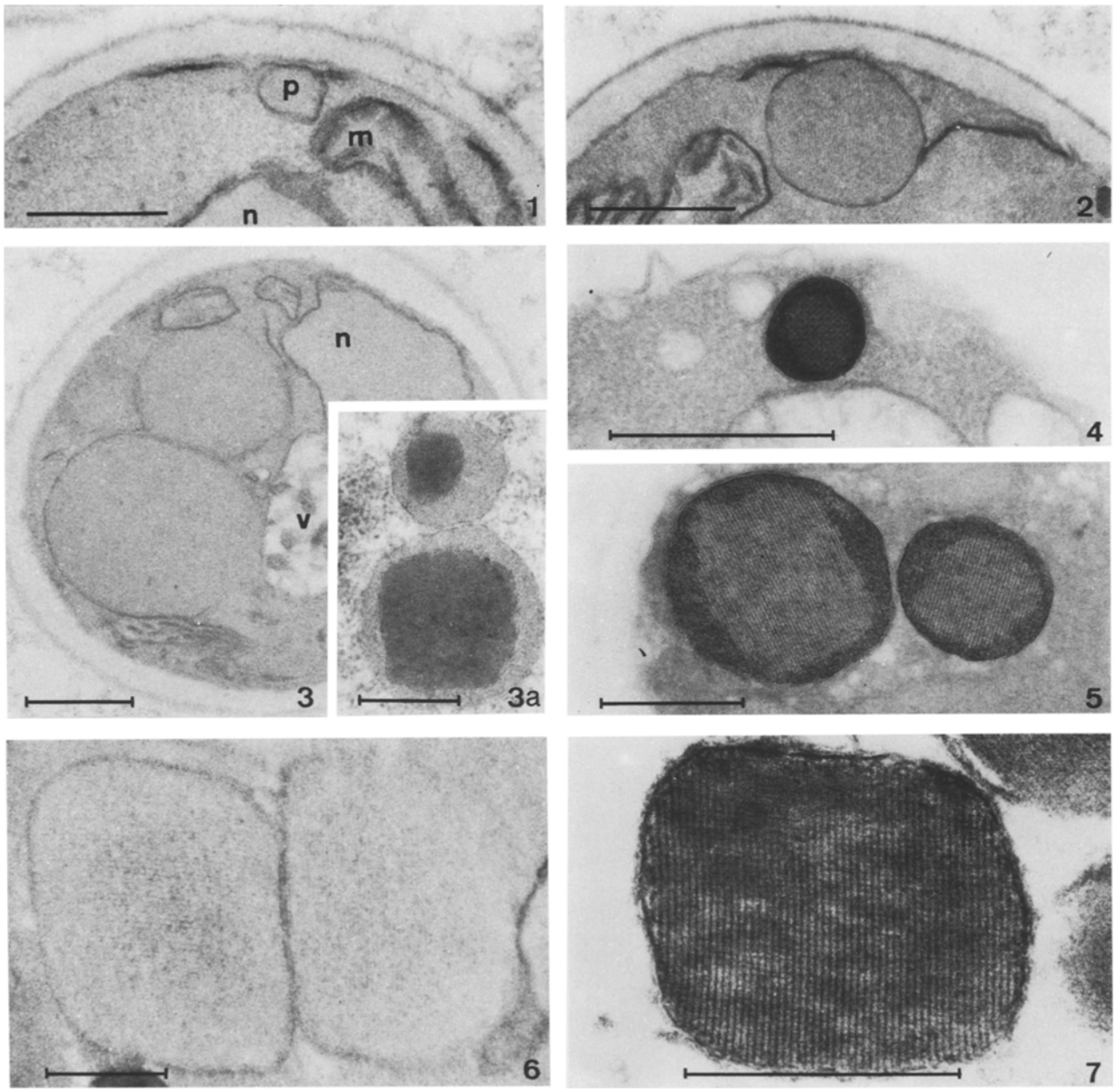
Morphometrical Analysis of Thin Sections. The average number of peroxisomes in thin sections of cells was estimated by at random counting (Veenhuis et al. 1978a). Volume densities of peroxisomes in the cytoplasm were estimated with the point counting technique according to Weibel and Bolender (1976). Student's *t*-test was used for statistical analysis.

Results

I. Development of Peroxisomes in Hansenula polymorpha During Growth in Media Containing Methanol and Methylamine

Growth of Organisms and Enzyme Profiles. When *H. polymorpha*, precultured on glucose/ammonium sulphate media was transferred into a medium containing methanol as the carbon source and methylamine as the nitrogen source, growth resumed after a lag of 1–2 h and was slightly slower than in cultures with methanol/ammonium sulphate. Enzyme assays revealed that the synthesis of alcohol oxidase and amine oxidase started already during the lag phase following the transfer. In addition the activity of catalase increased. Enzyme profiles of alcohol oxidase and catalase were essentially similar during growth in methanol/methylamine or methanol/ammonium sulphate media (grown as a control; Table 1). Amine oxidase activity was not detectable in cells grown in methanol/ammonium sulphate media. In chemostat-grown cells ($D = 0.088\,h^{-1}$), the activities of alcohol oxidase, amine oxidase and catalase were about two-fold higher than those detected in batch culture (Table 1).

Electron Microscopical Observations. Transfer of glucose-grown cells of *H. polymorpha* into media containing methanol and methylamine resulted in a rapid increase in the size of the peroxisomes originally present in these cells (Figs. 1 and 2) (Veenhuis et al. 1979a). Along with the increase in size a crystalline inclusion developed in the peroxisomal matrix. Peroxisomes in cells from the stationary phase of growth showed an almost spherical shape in thin sections and con-



The figures represent micrographs of cells of *H. polymorpha*, grown in methanol/methylamine medium, which were fixed/postfixed with $KMnO_4$, unless otherwise indicated. The marker represents 0.5 µm. Abbreviations: *m* = mitochondrion, *n* = nucleus, *p* = peroxisome, *v* = vacuole

Fig. 1. Detail of a cell from the exponential growth phase on glucose (OD_{663} = 1.0) demonstrating the typical appearance of a peroxisome, present in these cells

Fig. 2. Detail of a cell 6 h after transfer from glucose/ammonium sulphate into methanol/methylamine containing media, showing the increase in size of the peroxisome, originally present in the glucose-grown cell. Note the characteristic association of the organelle with strands of ER

Fig. 3. Survey of a cell grown in a batch culture taken from the stationary phase of growth. The inset shows a detail of a spheroplast to demonstrate the substructure of the peroxisomes present in cells from the stationary growth phase (glutaraldehyde-OsO_4/$K_2Cr_2O_7$)

Figs. 4 and 5. Details of spheroplasts, demonstrating alcohol oxidase activity after incubation with $CeCl_3$ and methanol in peroxisosomes present in cells 6 h after transfer to methanol/methylamine (Fig. 4) and methylamine oxidase activity after incubation with $CeCl_3$ and methylamine in cells from the stationary phase of growth (Fig. 5) (glutaraldehyde-OsO_4/$K_2Cr_2O_7$)

Fig. 6. Detail of a cell after a short time incubation with DAB and methylamine demonstrating the distribution of the initial reaction products throughout the peroxisomal matrix

Fig. 7. Micrograph of an isolated peroxisome, subjected to an osmotic shock, positively stained after incubation with $CeCl_3$ and methylamine (glutaraldehyde-OsO_4/$K_2Cr_2O_7$)

tained a large crystalline inclusion (Fig. 3). Completely crystalline, cubically shaped organelles, were only observed in chemostat-grown cells. Morphometrical analysis of thin sections of $KMnO_4$-fixed cells indicated that during growth of *H. polymorpha* on methanol, the presence of methylamine as a nitrogen source instead of ammonium sulphate did not result in a significant increase in the average number of peroxisomes per cell or their relative volume density (Table 1). There was, however, a significant effect on the size of the vacuole; when methylamine was used as the nitrogen source the volume

density of the vacuole had increased threefold (Table 1). The significance of this increase in size of the vacuole remains to be elucidated.

Cytochemical staining experiments, performed on spheroplasts prefixed with glutaraldehyde, revealed that alcohol oxidase and amine oxidase were both present in peroxisomes, which developed in the cells during the lag phase which occurred after transfer of cells from glucose/ammonium sulphate into methanol/methylamine media (Figs. 4 and 5). Staining of cells from the stationary phase of growth or of chemostat-grown cells revealed that all the individual peroxisomes contained activities of both enzymes. In addition to amine oxidase and alcohol oxidase also catalase activity was detected in the organelles. Time-dependent incubations of chemostat-grown cells with DAB and methylamine as the H_2O_2-generating substrate indicated that amine oxidase activity was present throughout the crystalline matrix of the organelles (Fig. 6). However, the periodicity of the crystalline matrix was similar to that observed in methanol/ammonium sulphate grown cells (Figs. 7 and 11). Cytochemical staining experiments, performed on isolated peroxisomes which had been subjected to an osmotic shock, showed that amine oxidase activity was still present in the crystalloids (Fig. 7). In such organelles catalase activity was no longer detectable.

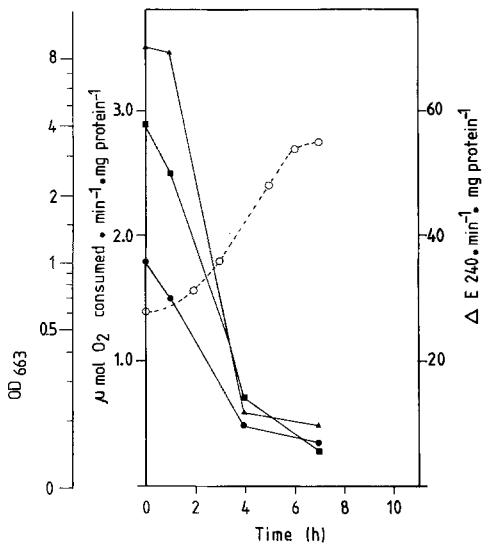


Fig. 8. Growth and enzyme profiles after transfer of chemostat-grown cells of *H. polymorpha* in media containing methanol and methylamine into batch cultures supplemented with glucose and ammonium sulphate. ○·····○ growth; ▲——▲ catalase activity; ■——■ alcohol oxidase activity; ●——● amine oxidase activity $\times 10^{-2}$

II. Transfer Experiments

During exponential growth of *H. polymorpha* in media containing glucose and ammonium sulphate both alcohol oxidase and amine oxidase are absent, while catalase is present in low activity (Eggeling and Sahm 1978; Veenhuis et al. 1979a; Egli et al. 1980; Zwart et al. 1980). This has prompted several groups of workers (Bormann et al. 1978; Veenhuis et al. 1978b) to study the fate of peroxisomes in cells grown in methanol-containing media after transfer of organisms to media with glucose as the carbon source. In these studies a rapid decrease in activity of typical peroxisomal enzymes associated with degradation of peroxisomes was observed which led us to postulate that peroxisomes whose physiological function was no longer required for growth were rapidly degraded. The present finding that *H. polymorpha* cells grown in media supplemented with methanol and methylamine contain peroxisomes in which alcohol oxidase and amine oxidase occur simultaneously allows further verification of this hypothesis.

Transfer of Methanol/Methylamine-Grown Cells into Glucose/Ammonium Sulphate Media. When cells of *H. polymorpha* grown under conditions where two peroxisomal enzymes were essential for growth were transferred into a medium where neither of these enzymes is required, the organism continued to grow without any detectable lag. The activity of both alcohol oxidase and amine oxidase in the cells decreased almost immediately, while the decline of the activity of catalase was initiated approximately 1 h after the transfer (Fig. 8). Four h after the transfer the activity of alcohol oxidase and catalase had decreased to approximately 10% of the original value, while in the same time interval amine oxidase activity diminished to 26% of the original activity. Electron-microscopical observations of $KMnO_4$-fixed cells revealed that the loss of enzyme activities from the cells was associated with a rapid degradation of the crystalline peroxisomes, present in the inoculum cells (Fig. 10). Budding of cells after the transfer was no longer associated with the migration of small crystalline peroxisomes from the mother cell into the developing bud which is so characteristic during budding of cells in methanol-

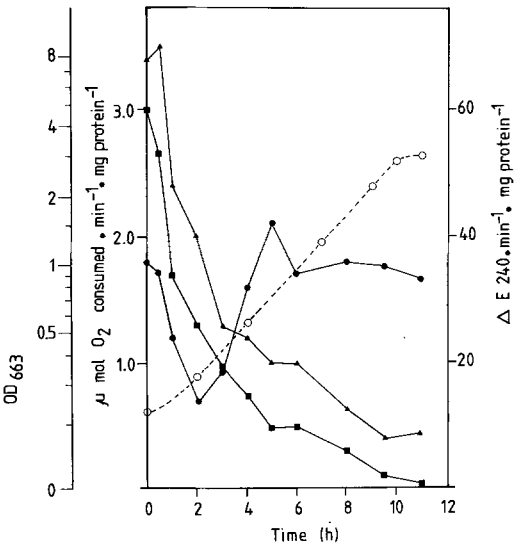


Fig. 9. Growth and enzyme profiles after transfer of chemostat-grown cells of *H. polymorpha* in media containing methanol and methylamine into batch cultures supplemented with glucose and methylamine. Symbols as in Fig. 8

containing media (Veenhuis et al. 1978a). In contrast, the first buds, which developed in the glucose medium, invariably contained a small peroxisome, typical for cells growing exponentially on glucose (Veenhuis et al. 1979a).

Transfer of Methanol/Methylamine-Grown Cells into Glucose/Methylamine Media. Growth and enzyme profiles during the first 2 h after transfer of methanol/methylamine-grown cells into glucose/methylamine media were similar to those observed after transfer of these cells into glucose/ammonium sulphate media (Fig. 9). However, 2 h after the transfer a distinct increase in amine oxidase activity was observed, whereas the decrease in alcohol oxidase and catalase activity continued. Amine oxidase activity reached its maximal value in the mid-exponential growth phase; this was followed by

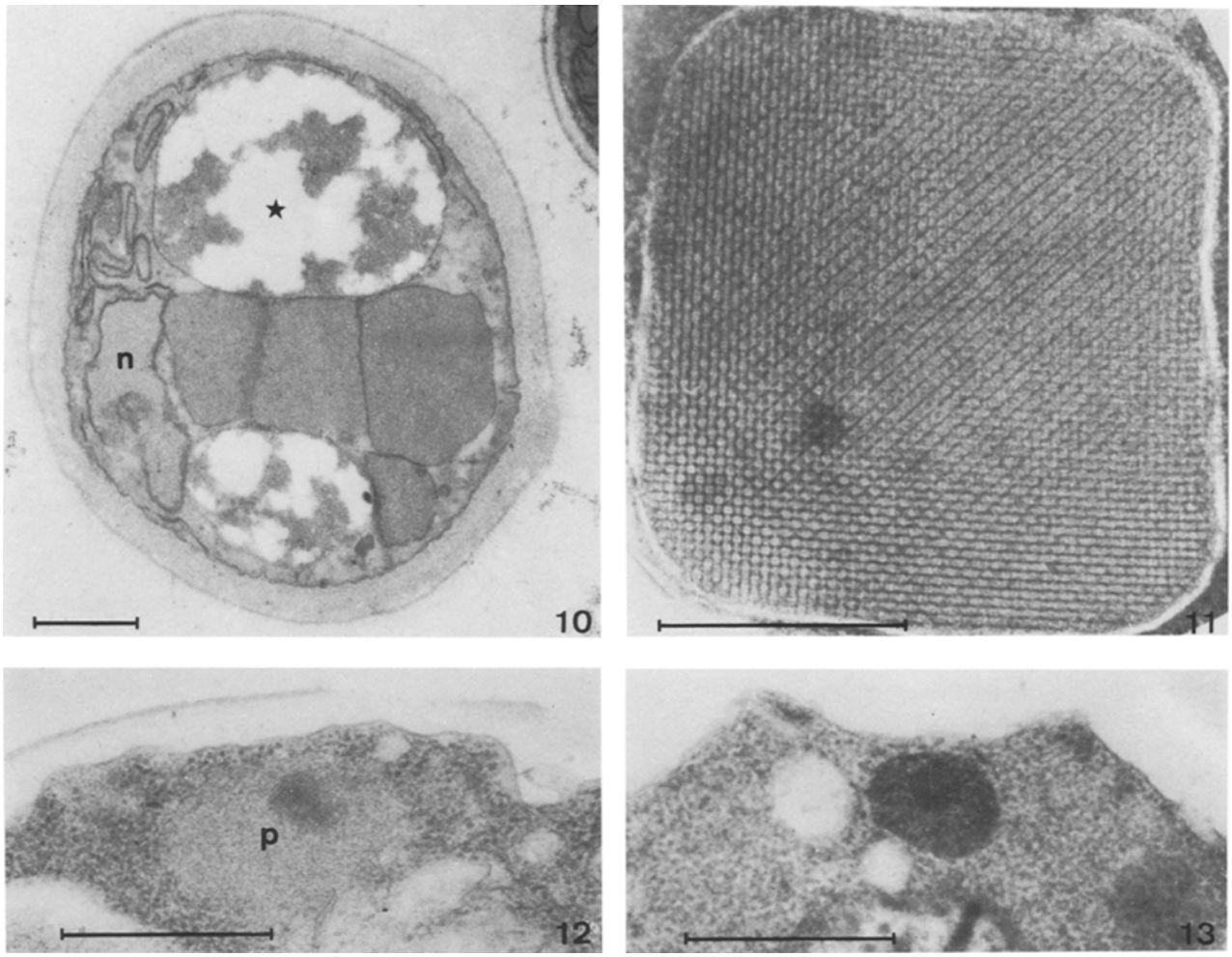


Fig. 10. Section through a chemostat-grown cell, 60 min after transfer of the cells into glucose and methylamine, showing desintegration of peroxisomes (*)

Fig. 11. Cryosection of a chemostat-grown cell on methanol/ammoniumsulphate, showing a peroxisome in which the individual molecules, arranged in a crystalline matrix, can be observed (lower left-hand corner)

Figs. 12 and 13. Details of spheroplasts from cells, 4 h after transfer from the chemostat to glucose/methylamine medium, showing a newly formed peroxisome with a small crystalline nucleus (Fig. 12). The presence of amine oxidase activity in these organelles was demonstrated after incubation with DAB and methylamine (Fig. 13) (glutaraldehyde-$OsO_4/K_2Cr_2O_7$)

a subsequent small decrease but the activity then remained approximately constant during prolonged cultivation (Fig. 9). Electron microscopical observations revealed that the observed loss of enzyme activities was associated with degradation of the peroxisomes present in the inoculum cells. However, 3 h after the transfer we observed besides continued degradation of peroxisomes which were present before transfer, the development of small peroxisomes in the cells (Fig. 12). Identical organelles were observed in developing buds. These organelles contained a small crystalline inclusion which is due to the presence of alcohol oxidase. Therefore, since the synthesis of alcohol oxidase is completely repressed under the conditions extant after the transfer, the organelles had probably derived from still intact organelles, originally present in these cells. During exponential growth the small peroxisomes increased in size. This process was not associated with any growth of the crystalline nucleus. Subsequently, the number of peroxisomes increased by a process of division (Veenhuis et al. 1979a). In these small organelles a crystalline inclusion was not observed. Cytochemical staining techniques indicated that both amine oxidase and catalase were present throughout the matrix of these organelles (Fig. 13). Alcohol oxidase activity was absent.

Effect of Substitution of Methylamine by Ammonium Ions During Growth on Methanol. This was investigated in a methanol-limited chemostat by changing the inflowing medium from one containing methanol and methylamine to one containing methanol and ammonium sulphate as the nitrogen source. The results obtained indicated that the synthesis of amine oxidase was repressed immediately after the transfer. Inactivation of existing amine oxidase probably did not occur since the decrease of activity in the culture could be accounted for by dilution as a result of growth and washout (Table 1). In contrast to the decrease of amine oxidase activity, alcohol oxidase activity had slightly increased after 3 volume changes while catalase activity remained approximately constant during prolonged cultivation.

Morphometrical analysis revealed that the volume density of the peroxisomes had increased after the transfer (Table 1). Since the average number of peroxisomes remained approximately constant the increase in volume density must be explained by an increase in size of individual organelles. Along with the observed increase in volume density of the peroxisomes, the volume density of the vacuole, present in the cells, decreased (Table 1).

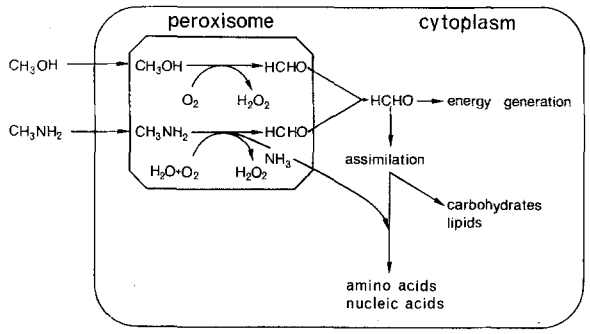


Fig. 14. Schematic representation of the significance of peroxisomes in the initial oxidation of methanol and methylamine in *H. polymorpha*. The products of these obligatory peroxisome-born reactions can be used by the cells for energy generation, carbon assimilation and nitrogen assimilation

Discussion

The results of the biochemical and electron microscopical experiments reported above indicated that growth of the yeast *Hansenula polymorpha* in media containing methanol as the carbon source and methylamine as the sole nitrogen source was associated with the presence of a number of peroxisomes in the cells, which contained catalase, alcohol oxidase and amine oxidase. These results provide the first example of peroxisomes which play a key role in the concurrent metabolism of both the carbon (Roggenkamp et al. 1975) and the nitrogen source (Zwart et al. 1980) for growth in yeasts (Fig. 14). In the course of our experiments it was observed that the development of these organelles, their ultimate shape and number and their substructure varied with growth conditions in a manner similar to that described for methanol/ammonium sulphate-grown cells (Veenhuis et al. 1978a, 1979a).

The cytochemical staining experiments indicated that amine oxidase may form an integral part of the crystalloid present in the peroxisomes of cells grown in methanol/methylamine media. In previous papers papers we have shown that the development of crystalloids in peroxisosomes of methanol-grown cells of *H. polymorpha* was strictly dependent on the synthesis of alcohol oxidase (Veenhuis et al. 1979a, 1979b). Amine oxidase is probably not involved in the formation of a crystalloid, since these structures were absent in glucose/methylamine-grown cells of *H. polymorpha* (Zwart et al. 1980). However, amine oxidase activity is present in the crystalloids of methanol/methylamine-grown cells and had not leaked from isolated organelles, which had been subjected to an osmotic shock in a way characteristic for catalase (Veenhuis et al. 1978a). Therefore the sub-structural organization of peroxisomes of *H. polymorpha* must be different from that of the crystalloids in peroxisomes of methanol-grown *Kloeckera* sp. no. 2201 since these structures were reported to be composed of repeating units of one alcohol oxidase and one catalase molecule (Osumi et al. 1979).

The transfer experiments indicated that peroxisomes, present in methanol-limited chemostat-grown cells of *H. polymorpha*, were subject to rapid degradation after transfer into glucose-containing media. Although the mechanism of degradation is still unclear, this process is probably initiated by fusion of the vacuole or vacuolar vesicles with the peroxisomes to be degraded (Veenhuis et al. 1978b), followed by hydrolysis of the peroxisomal proteins by proteolytic enzymes which are known to be exclusively present in yeast vacuoles (Wiemken et al. 1979). When cells were transferred to conditions where alcohol oxidase was no longer required because methanol had been replaced by glucose, but amine oxidase was still essential for growth due to the presence of methylamine as the sole nitrogen source, degradation of peroxisomes was observed as in earlier experiments (Veenhuis et al. 1978b). However, this degradation of the crystalline peroxisomes was now associated with the development of new peroxisomes which started approximately 3 h after the transfer as a result of the synthesis of amine oxidase in these cells. Judging from the rate of amine oxidase synthesis and the rapid development of these newly formed organelles, it is suggested that part of the amino acids, formed in the desintegrating peroxisomes by proteolysis, are used for the synthesis of amine oxidase. Also, they may contribute to the cell's nitrogen supply during the first hours of growth since amine oxidase activity decreased in this time interval to a value which could no longer account for the observed rate of growth of the organism.

Acknowledgements. We wish to thank J. Zagers for skillful assistance in preparing the figures and the electron micrographs and Dr. J. P. van Dijken for his interest and stimulating discusions. K. B. Zwart was supported by the Foundation for Fundamental Biological Research (BION), which is subsidized by the Netherlands Organization for the Advancement of Pure Research (ZWO).

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Received July 2, 1980